Analysis of Enzyme Activities of the Gut Bacterial Communities in *Labeo rohita* fed Differentially Treated Animal Fleshing Diets

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Abstract

The present investigation includes isolation, enumeration and comparison of the gut microbial flora of *Labeo rohita* fed differentially treated Animal Fleshing (ANFL) as a sole protein source in their diets and related increase in enzyme secretion. ANFL is the proteinaceous tannery solid waste generated during leather processing and inclusion of ANFL in aqua feed will pave way for the novel alternative cheaper protein source in replacement of fish meal. Six experimental diets were formulated with differentially processed ANFL. The isolates from the gut extract were qualitatively screened and quantitatively assayed for amylase, cellulase, lipase and protease activities. The total culturable bacterial count (10×10^7 CFU/g) as well as the proteolytic bacterial count (27×10^6 CFU/g) was high in the fish gut fed with diet 5 containing the fermented ANFL. Almost all the bacterial isolates possesses protease activity in which strains (FF5, CF3and CF4) isolated from the diet 5 (287U) and 7 (282U) exhibited highest activities. Maximum cellulase (FF2), amylase and lipase (RF6) activity were observed in the strains isolated from the diets 5 and 1 (78U, 186U and 97U). This study proves the existence of diet dependent enzyme producing bacterial community in the gut of *Labeo rohita* through SEM analysis and the information generated from the present study might contribute towards utilization of enzyme producing bacterial isolates as probiotic and in better feed formulations at low cost for rohu incorporating the tannery solid waste ANFL as a protein source.

Introduction

The micro-environment of the digestive tract of fish confers a favorable growth for the microorganisms [1-3] by providing ecological niche for these organisms [4]. The microbiota of fish has been shown to be highly dependent on the bacterial colonization during early development, environmental conditions, nutrient composition and dietary changes [5,6,62]. These microorganisms grow upon the food absorbed by the host animal, digestive secretions and fragments scaled off the mucosal epithelium [7] and have a better chance of survival [8].

The indigenous microflora of fish in aquaculture has previously been studied for several purposes [19,21,22].and differs from ovary skin and liver [71]. Previous studies include descriptions of microbial spoilage [9,72], relationship between environment and fish microflora [10], monitoring of changes in fish farms [11], nutritional role of the intestinal flora [12] and the antibiotic resistance profile of the indigenous flora [13]. Preceding research suggests that digestive enzymes present in fish digestive tract can elucidate some aspects of their nutritive physiology [73] and thus be supportive to develop nutritional strategies for fish feeding and diet formulation [14-17].

In general, the bacterial flora of the gastrointestinal tract with diversified enzymatic potential plays a vital role in major part of the metabolism of the host animal [18].From the studies made so far, it appears that different species of fish and crustaceans have a specific resident gut microbiota [60,62,85].and the isolated species include *Acinetobacter* spp, *Enterobacter* spp. and *Pseudomonas* spp. in trout (*Oncorhynchus* sp.); *Aeromonas* spp., *Flavobacterium* spp. and *Lactobacillus* spp. in Arctic charr (*Salvelinus alpines*) [58]. *Enterovibrio* spp. from the intestinal tract of turbot (*Scophthalamus maximus*)[59]. *Vibrio* spp*., Bacillus* sp*., Pseudomonas* sp*., Photobacterium* sp. and *Plesiomonas* sp. in white shrimp, *Fenneropenaeus* (*Penaeus*) *indicus*

[60]. *P. glacincola* in the hind-gut of Arctic charr [6]. and Atlantic cod [22]. *Vibrio* species from gut of halibut larvae [69] and Atlantic cod [74,22]. P. phosphoreum farmed Norwegian salmon [65]. and UK halibut [60]. *Providencia* sp. and *Shewanella* sp.[63]. However, the information regarding the enzyme producing intestinal bacteria related to feed in fish is scarce [16].

The inter-relationships between the microbiota and the host are clearly important as the health and imbalance between these systems appears to drive a wide range of mucosal and systemic immune-mediated disorder [26,27]. Several authors revealed the presence of microbiota in fish gut [19-22]. and enzyme producing bacteria in the digestive tract of *L. rohita* [23,24]. The use of such beneficial bacteria has a long tradition in the animal husbandry [25]. However, the research based on gut microbial population in fish species and digestive enzymes related to animal fleshing feed source, their significance as a means to determine assimilating capacity is perhaps nil.

Animal protein sources present good essential amino acids balance [76].but costs and heterogeneous composition repeatedly limit their use, and the use of alternative sources is required [77]. Hence, amino acid rich proteinaceous tannery solid waste animal fleshing was used as a protein source in fish feed formulation. India is one of the leading

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countries in freshwater aquaculture. In 1998, the total production of freshwater fish was1.7 million tons of which carps contributed about 87%. Rohu (*Labeo rohita*) is the most important Indian major carp that enjoys high consumer preference in many states with high protein conversion ratio [75]. Hence, the focal theme of the present work was to evaluate the enzyme producing microbiota and activities of enzymes in the digestive tract of rohu, *Labeo rohita* fed differentially treated animal fleshing incorporated diets.

Materials and Methods

Acclimatization of fish to experimental conditions

Labeo rohita fingerlings (average weight 5g ±0.25g) were obtained from nearest farm in Chennai (India) and acclimatized in 1000 L cylindrical tanks filled with dechlorinated tap water for 15 days with control feed. Three hundred and fifty fingerlings were selected for the study and divided into six experimental groups and one control. Each group of 50 fingerlings was again divided into two equal duplicate subgroups. Fish were fed twice daily (9:00 a.m. and 17:30 p.m.) to apparent satiation. The fish were fed 3% of their body weight twice a day. The limed animal fleshing was collected from a tannery processing raw goat skins/cow hides into leather in Chennai. Briefly, the animal fleshing was subjected to various optimized treatments (data not shown) such as microwave for 60 minutes, ozonation for 60 minutes, soaking in H_2O_2 for 60 minutes, fermentation with bacterial mixed culture from the fish gut and autoclaving (Table 1). The impact of nutritional effects on gut flora and gut enzyme was studied on 60th day.

Microbiological Examination of Gut Flora

Fish from each experimental set was collected, after 36 hrs of starvation. The fish was sacrificed and surface sterilized using 1% (w/v) iodine solution. For microbiological examination and enzyme analysis the intestine was dissected on ice slab in sterile condition. The intestine samples were then homogenized in a surface sterilized mortar and pestle. The homogenized samples were serially diluted (ten folds) with sterile chilled buffered saline solution. Diluted samples (0.1 ml) were spread aseptically within a laminar airflow on sterilized Schaedler HiVegTM Agar (Hi media, India) to determine the total culturable gut heterotrophic bacterial population. Spread plate technique was used to

isolate and enumerate protease, cellulase, amylase and lipase producing bacterial population, diluted samples (0.1 ml) was poured on skim milk agar (SMA), Carboxy methyl cellulose agar (CMC), Starch agar (SA) and Tween20 agar (TA) plates, respectively. Culture plates were incubated at 37°C for 48 hours and were subsequently examined for the development of bacterial colonies. It was assumed that the micro flora, which had formed colonies on the SA plate, had amylolytic activity, CMC- cellulolytic, SMA -proteolytic and TA plates- lipolytic activities. Colony numbers per unit sample volume of gut homogenate were determined by multiplying the number of colonies formed on each plate by the reciprocal of dilution. The well separated colonies producing various extracellular enzymes with apparently different morphology were streaked separately on Tryptone Soy Agar (TSA) (Hi Media, India) plates to obtain pure cultures [74]. Single, isolated colonies from the streaked plates were transferred to TSA slants for further study. Chemicals used for analysis were procured from Merck and Hi Media, India. No conflict of interest existed during the entire study period.

Qualitative assay for enumeration of enzyme producing bacteria

The intensity of extracellular enzyme production by the pure cultures was determined on agar plates with selective media. For extracellular amylase production, the samples were inoculated on SA plates and incubated at 37°C for 48 h. The culture plates were then flooded with 1% Lugol's iodine solution [56]. formation of transparent zone surrounding the colony indicated amylase activity. Similarly, for extra-cellular protease, the samples were inoculated on SMA plates and incubated at 37°C for 24 h. The appearance of a clear zone around the colony confirmed the proteolytic activity. For determination of cellulase production, the samples were grown on CMC plates at 37°C for 24 h and flooded with 0.1% Congo red dye and destained with 1M sodium chloride [57]. Congo red selectively binds with unhydrolyzed CMC. Appearance of clear halo due to the presence of hydrolyzed CMC surrounding bacterial colony indicated cellulase production in the medium. For assaying lipase activity the samples were inoculated on the TA plates. Formation of calcium laurate white crystals due to the reaction between the fatty acids released and $CaCl_2$ present in the medium around the colonies confirm the lipase activity of the colony.

Media composition

Schaedler HiVegTM Agar(g L^{-1}): Hiveg hydrolysates, 5.67; Hiveg peptone No.3, 5.00; Papaic digest of soyabean meal, 1.00; Yeast extract, 5.00; Dextrose, 5.83; Sodium chloride,1.67;Dipotassiumhydrogen phosphate, 0.83; Tris hydroxymethyl aminomethane, 3.00; L-cysteine, 0.40; Ferric pyrophosphate, 0.01; Agar, 15; pH 7.6; TSA medium (HI-Media, Chennai, India) (g L^{-1}): Pancreatic digest of casein, 15; Papaic digest of soyabean meal, 5; NaCl, 5; Agar, 15; pH 7; SA medium (g L^{-1}): Beef extract, 5; Peptone, 5; NaCl, 5; Starch (soluble), 2; Agar, 20; pH 7; CMC medium (g L^{-1}): Beef extract, 5; Peptone, 5; NaCl, 5; Carboxymethyl cellulose, 2; Agar, 20; pH 7; SMA (g L^{-1}): Skim milk 20; NaCl 5; Agar 20; pH 7; TA (gL-1): Tween 20 -10, Peptone 15; NaCl 5; $CaCl_2$ 1; pH 7.

Quantitative enzyme assay of gut and the bacterial isolates

Fish gut from each feed trial was aseptically removed on 60th day after 36 h starvation for quantitative assay of cellulase, amylase, and protease and lipase activity of gut enzyme. The gut was homogenized in 10 ml chilled buffered saline to make a suspension and then centrifuged at 4500 rpm for 15 min. The clear supernatant was used

Experimental diets	Rice Bran (g)	Wheat Flour (g)	Oil Cake (g)	Sunflower Oil (g)	Protein Source (35 g)
DIET 1	20	20	10	1	Raw ANFL (rf)
DIET 2	20	20	10	1	Microwave treated ANFL (mf)
DIET 3	20	20	10	1	Ozone Treated ANFL
DIET 4	20	20	10	1	H_2O_2 treated ANFL (hf)
DIET 5	20	20	10	1	Fermented ANFL (ff)
DIET 6	20	20	10	1	Autoclave ANFL (af)
CONTROL	20	20	10	1	Fish meal (cf)

Vitamin premix mg/g : retinol palmitate 400,000IU, thiamine 5,riboflavin 4,niacin 20,.folic acid,2, pyridoxine,6,cyanocobalamine 5, ascorbic acid,12,cholecalciferol 55,000IU, tocoferol 3, biotin 0.3,choline chloride 90,pantothenic acid ,10. *Mineral premix* for 100g calcium phosphate dibasic, 7.35; citric acid,0.03; ferrous sulphate 50 mg magnesium oxide 3; manganese sulphate 0.7; potassium phosphate dibasic,9 ;sodium chloride

Table 1: Aqua feed composition.

for assaying various enzyme activities. Selective broth media were used as production media for a quantitative assay of amylase, cellulase, and protease and lipase production of selected bacterial isolates. A loopful of selected strain was inoculated into Tryptone soya broth and incubated for 24 h at 37ºC. The liquid production medium about 25 mL was inoculated with 2% of the inoculums obtained from seed culture. The culture flasks were incubated for 48 h at 37 ± 1ºC. After incubation, the contents were centrifuged at 4500 g for 15 min, at 4ºC and the cell-free supernatant was used for enzyme assay. Ten fish were harvested from each experimental setup for further analysis.

Amylase assay

Amylase activity was assayed by the dinitrosalicylic acid (DNSA) method based on the estimation of reducing sugars at 540 nm using maltose as the standard (Bernfeld 1955). 1% of starch in sodium phosphate buffer (0.1M, pH 7.0) acted as substrate for the assay. One amylase unit was defined as the amount of enzyme per milliliter of sample that released one microgram (μg) reducing sugar (maltose) per minute.

Cellulase assay

Cellulase activity was measured according to the method of Denison and Kohen (1977) using 1% CMC in potassium phosphate buffer (0.1M, pH 7.0) as substrate. The production of reducing sugar (glucose) from CMC substrate as a result of cellulolytic activity was measured at 540 nm by dinitrosalicylic acid method using glucose as the standard. One cellulase unit was defined as the amount of enzyme per milliliter of sample that released one microgram (μg) of reducing sugar (glucose) per minute.

Protease assay

Protease activity was determined according to Kunitz caseinase assay method using 1% Casein in sodium phosphate buffer (0.1M, pH 7.0) as substrate. One unit of enzyme activity represents the amount of enzyme required to liberate 1 μg of tyrosine per milliliter sample under standard assay conditions.

Lipase assay

Lipase assay was carried out based on titrimetric method using olive oil as a substrate and polyvinyl alcohol (PVA) as an emulsifier [28].

Scanning Electron Microscopy (SEM)

The gut sections designated for SEM analyses were processed according to Ghosh et al [29]. dehydrated with a graded ethanol series and subsequently freeze dried .The dried cells were sputtered coated with gold, and finally photographed by a scanning device attached to a JEOL JM – 5600 electron microscope at 20 kV accelerating voltage for an electron beam of wavelength 5–6 nm.

Statistical Analysis

Statistical analysis of the experimental data was made by analysis of variance (ANOVA) followed by Scheffe's F-test for multiple comparison [30].

Results

Analysis of the bacterial flora in the gastrointestinal tract of the experimental fish showed that the total culturable aerobic gut bacterial population on Schaedler HiVeg TM Agar plate were in the order of fermented ANFL (100000×10^3 CFU/g gut tissue) > control > microwave treated > autoclaved ANFL > raw ANFL. The total aerobic bacterial population was found to be least in ozone treated (120×10^3 CFU/g gut tissue) and H_2O_2 treated ANFL (15×10^3 CFU/g gut tissue) (Table 2).

While enumerating specific enzyme producing bacterial flora, it was observed that the amylolytic strains were highest in microwave treated (4200×10^3 CFU/g gut tissue) and fermented ANFL (3300×10^3 CFU/g gut tissue) incorporated diets, followed by raw autoclaved > ozone > H_2O_2 treated ANFL incorporated diets. The cellulolytic population exhibited maximum activity in fermented (48000×10^3 CFU/g gut tissue) and autoclaved ANFL (30000×10^3 CFU/g gut tissue) followed by microwave > raw> ozone > H_2O_2 treated ANFL. Lipolytic bacterial flora were detected in the experimental fish and the maximum population density was recorded in fermented (3700×10^3 CFU/g gut tissue) and autoclaved ANFL (3400×10^3 CFU/g gut tissue) followed by raw > microwave treated > ozone > H_2O_2 treated ANFL. Proteolytic bacterial flora were found in all the experimental fish and the maximum count was observed in the gut of rohu (*Labeo rohita*) fed with fermented (27000×10^3 CFU/g gut tissue) and microwave treated (24000×10^3 CFU/g gut tissue) ANFL followed by raw, autoclaved > ozone > H_2O_2 treated ANFL. On comparison with control the protease producing bacterial population in the experimental feeds containing fermented and microwave treated ANFL, were quiet high. Lipase producing bacterial population was similar to control samples in fermented and autoclaved ANFL fed fish guts and low in all other diets (Table 3).

The intensity of extracellular enzyme activity of the intestinal extract of *Labeo rohita* gut from each experimental feed was assayed quantitatively and represented as shown in Figure 1. All the diets exhibited higher protease activity. Peak specific amylase and cellulase activities were exhibited by gut extracts of microwave and fermented

Experimental feed (Protein Source) Diets	Total Bacterial Count (CFU × 10³ /g gut tissue)	Cellulolytic Bacterial Count (CFU × 10³ /g gut tissue)	Amylolytic Bacterial Count (CFU × 10³ /g gut tissue)	Proteolytic Bacterial Count (CFU × 10³ /g gut tissue)	Lipolytic Bacterial Count (CFU × 10³ /g gut tissue)
1.Raw ANFL	28000[d]	1500[e]	300[e]	20000[c]	230[d]
2.Microwave ANFL	70000[b]	12000[d]	4200[a]	24000[b]	200[e]
3.Ozonated ANFL	120[e]	120[f]	10[g]	10[f]	70[f]
4. H₂O₂ treated ANFL	15[f]	13[g]	15[f]	15[e]	10[g]
5.Fermented ANFL	100000[a]	48000[a]	3300[b]	27000[a]	3700[b]
6.Autoclave ANFL	70000[b]	30000[b]	2000[c]	15000[d]	3400[c]
Initial (0th day)	40000[c]	23000[c]	1000[d]	20000[c]	4000[a]

Results are means of triplicate treatments. Values with different superscript in the same column are significantly different.

Table 2: Aerobic bacterial count in the gut of rohu fed with experimental diet.

Experimental diets	Bacterial strains	Amylase activity	Cellulase activity	Protease activity	Lipase activity
1.Raw ANFL	RF3	++	+++	+++	+++
	RF5	+++	+++	+++	++
	RF6	++++	++	+++	++++
2.Microwave treated ANFL	MF1	—	+++	++++	++
	MF2	+++	+++	+++	+++
	MF3	+++	—	+++	++
	MF6	++++	+++	++++	—
3.Ozone treated ANFL	OF2	++	++	+++	—
	OF3	+++	++	+++	++
	OF5	+++	+++	+++	++
4.H2O2 treated ANFL	HF4	++	++	+++	++
	HF5	—	+++	+++	++
5.Fermented ANFL	FF1	++	+++	+++	+++
	FF2	++++	++++	++++	—
	FF3	+++	+++	++++	++
	FF4	+++	++++	++++	+++
	FF5	+++	—	++++	+++
6.Autoclaved ANFL	AF1	++++	+++	++++	—
	AF3	++	+++	+++	+
	AF4	+++	—	++++	+++
	AF5	+++	+++	+++	+++
7.Control	CF2	+++	++	++++	++
	CF3	++	++	++++	+++
	CF4	+++	—	++++	+++

'+' sign indicates the intensity of enzyme production (zone diameter of 4mm). ++++, very high; +++, high; ++ moderate; +, low; —, nil.

Table 3: Qualitative extracellular enzyme activity of bacterial strains isolated from *Labeo rohita* fish gut fed with experimental diets.

ANFL fed fish than other diets. Specific activity of lipase was found to be highest in the fermented ANFL fed fish and maximum protease activity was observed in fermented ANFL and Microwave treated ANFL fed fish. The protease and lipase activity of the fermented ANFL fed rohu gut extract was similar to control.

The intensity of extracellular enzyme production by the bacterial strains isolated from the gut of rohu was assayed qualitatively (Table 3) in which each '+' indicates a zone diameter of 4mm (radius–2mm). Among these isolates, eight amylase, eight cellulase, nine lipase and fourteen protease producers were selected for quantitative enzyme assay. Peak cellulase (Figure 2) and amylase (Figure 3) activities were exhibited by the bacterial strains FF2 and RF6 isolated from
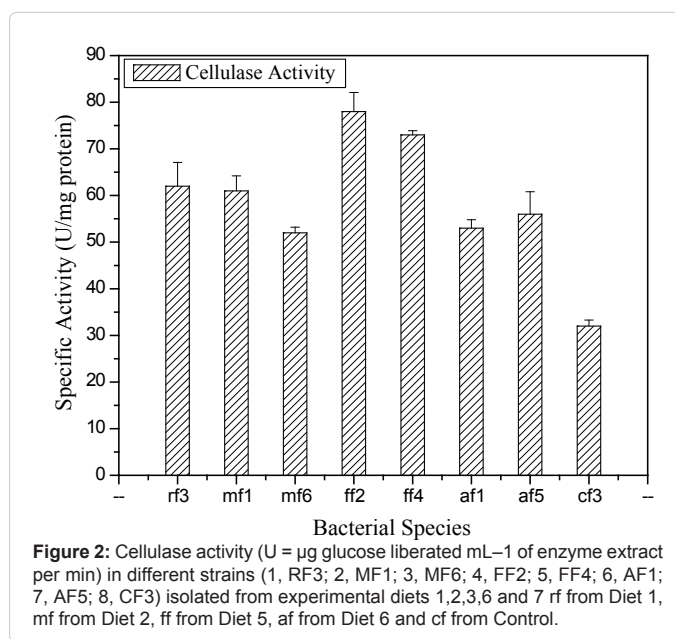

Figure 2: Cellulase activity (U = µg glucose liberated mL–1 of enzyme extract per min) in different strains (1, RF3; 2, MF1; 3, MF6; 4, FF2; 5, FF4; 6, AF1; 7, AF5; 8, CF3) isolated from experimental diets 1,2,3,6 and 7 rf from Diet 1, mf from Diet 2, ff from Diet 5, af from Diet 6 and cf from Control.
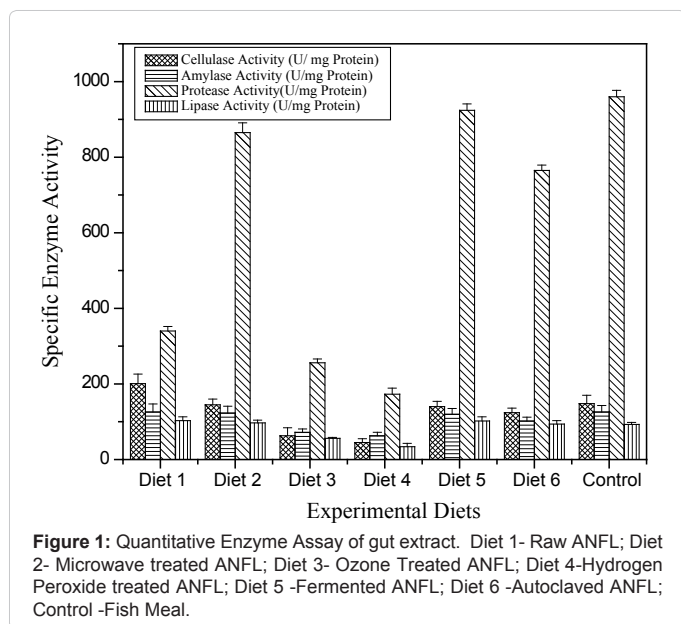

Figure 1: Quantitative Enzyme Assay of gut extract. Diet 1- Raw ANFL; Diet 2- Microwave treated ANFL; Diet 3- Ozone Treated ANFL; Diet 4-Hydrogen Peroxide treated ANFL; Diet 5 -Fermented ANFL; Diet 6 -Autoclaved ANFL; Control -Fish Meal.

experimental diet groups1 and 5 respectively. Maximum protease (Figure 4) activity was observed in the strain FF5 isolated from experimental diet 5, followed by the strains CF3 and CF4 isolated from the control groups. The strain rf6 from experimental diet 1 showed highest lipase activity followed by ff5 and cf4 (Figure 5). In the present study, proteolytic bacteria were detected in the gut of all the fish examined and maximum density of proteolytic bacterial population was observed in the experimental diets 5 and 2 (2.7×10^7 and 2.4×10^7 CFU· g^{-1} intestinal tissue). However, assay of extracellular protease activity of the bacterial isolates showed highest value in FF5 (287 U), a strain isolated from the diet 5 fed fish gut. SEM analysis of *Labeo rohita* gut (Figure 6), the arrow marks indicates the position of the bacterial cells attached to the gut wall.

Discussion

The factor contributing to the bacterial assemblage is diet [71]. Protein is the dietary macronutrient and energy source whose requirement is prioritized in nutritional studies, either because it represents the highest fish feed cost, or greatly affects specific growth rate [78,80].Digestive enzyme activities of fish are associated with innate feeding habit and diet composition [81,82]. Hence, the study of digestive enzymes is an essential step towards understanding the mechanism of digestion and how organisms adapt to changes in the nutritional environment [83,84]. The proteinaceous ANFL generated from Leather industry after various treatments physical (autoclave and microwave), chemical (H_2O_2 and O_3) and microbial fermentation were
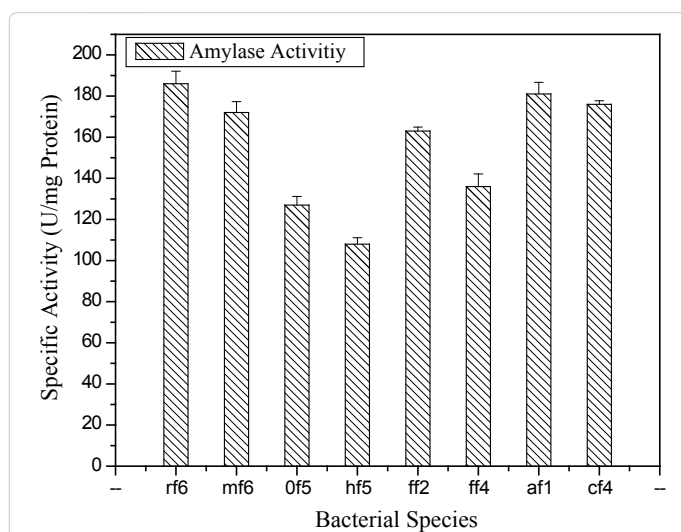

Figure 3: Amylase activity (U = µg maltose liberated · mL^{-1} of enzyme extract per min) in different strains (1, rf6; 2, mf6; 3, of6; 4, hf5; 5, ff2; 6, ff4; 7, af1; 8, cf4) isolated from the experimental diets rf from Diet 1, mf from Diet 2, of from Diet 3, ff from Diet 5, af from Diet 6 and cf from Control.
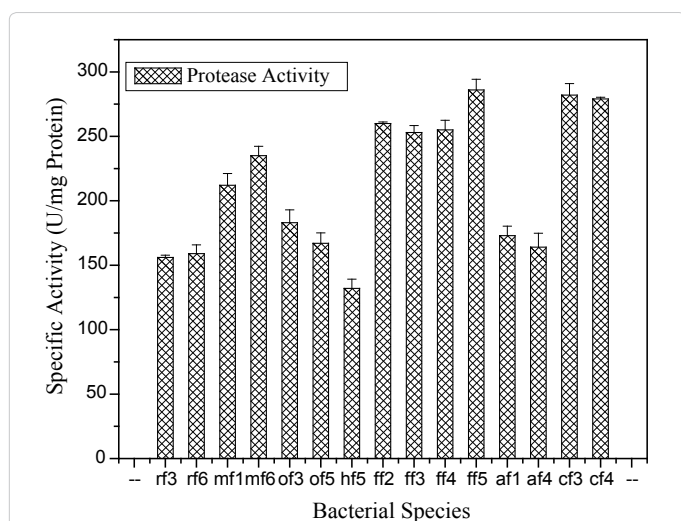

Figure 4: Protease activity (U = µg tyrosine liberated · mL−1l of enzyme extract per min) exhibited in different strains (1, rf3; 2, rf6; 3, mf1; 4, mf6; 5, of3; 6, of5; 7, hf5; 8, ff2; 9, ff3; 10,ff4; 11, ff5; 12, af1; 13, af4; 14, cf3; 15, cf4 ;) isolated from all the experimental diets respectively. rf from Diet 1, mf from Diet 2, of from Diet 3, ff from Diet 4, ff from Diet 5, af from Diet 6 and cf from Control.

Figure 5: Lipase activity (U= amount of fatty acids released mL^{-1} of enzyme extract per minute) in the selected strains (1, rf6; 2, mf2; 3, of3; 4, hf5; 5, ff4; 6, af5; 7, cf3; 8, cf4 ;) from all the experimental diets respectively. rf from Diet 1, mf from Diet 2, of from Diet 3, hf from Diet 4, ff from Diet 5, af from Diet 6 and cf from Control.
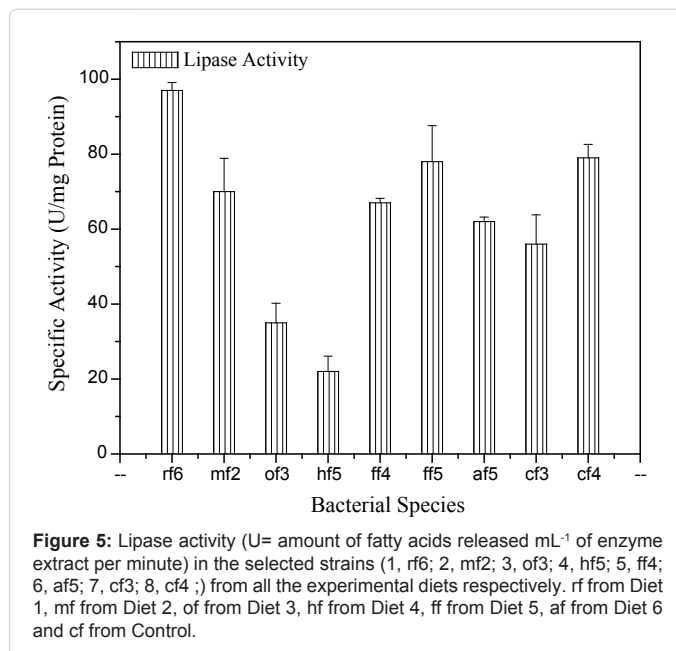
included in diets of *Labeo rohita* to analyze the ANFL incorporated diet assimilating bacterial population with enzyme producing capacity. The processing techniques of ANFL includes heat treatment to obtain sterile product as defined by Codex Alimentarius (FAO) for the reduction in viable count in ANFL [31]. chemical process through ozonation and hydrogen peroxide treatment which are strong oxidizers that kills micro-organisms by blocking their enzyme control system and deodorizes both gaseous and particulate matter by a oxidative mechanism and removes the contaminants which can destroy the protein resulting in obtaining better level of protein [32-34] and microbial fermentation with mixed culture which is a best approach to obtain the desired product [35].

The gastrointestinal (GI) tract of fish is a complex ecosystem possessing a dense bacterial population levels consisting of aerobic, facultative anaerobic and obligate anaerobic bacteria [64-69].that can be defined as either autochthonous (indigenous) or allochtonous (transient) depending upon its ability to adhere and colonize the mucus layer in the digestive tract [36,37,70].

In the present investigation, the presence of a considerable population of bacterial flora in the gastrointestinal tracts of the fish species is confirmed through SEM analysis and supports the earlier reports that the bacteria entering along with the diet of fish during ingestion may adapt themselves in the gastrointestinal tract to form a symbiotic association and may be considered to have possible contribution in nutritional process of fish via extracellular bacterial enzymes. Moreover, certain strains of bacteria isolated from the gut extract exhibited quantitative amount of amylolytic, cellulolytic, lipolytic and proteolytic activities [15,38-41]. The Fermented ANFL increases protease producing bacterial population on gut and thereby aids in the digestion of proteinaceous diet. On the other hand, the H_2O_2 and Ozone (oxidizing and bactericidal agent) treated ANFL had the least bacterial count and low enzyme activity. The complexity and intact structure of raw ANFL minimizes the gut microbial protease activity of fish fed with diet 1 but cellulase and amylase activity were seen due to the presence of rice bran and wheat flour which are plant based natural
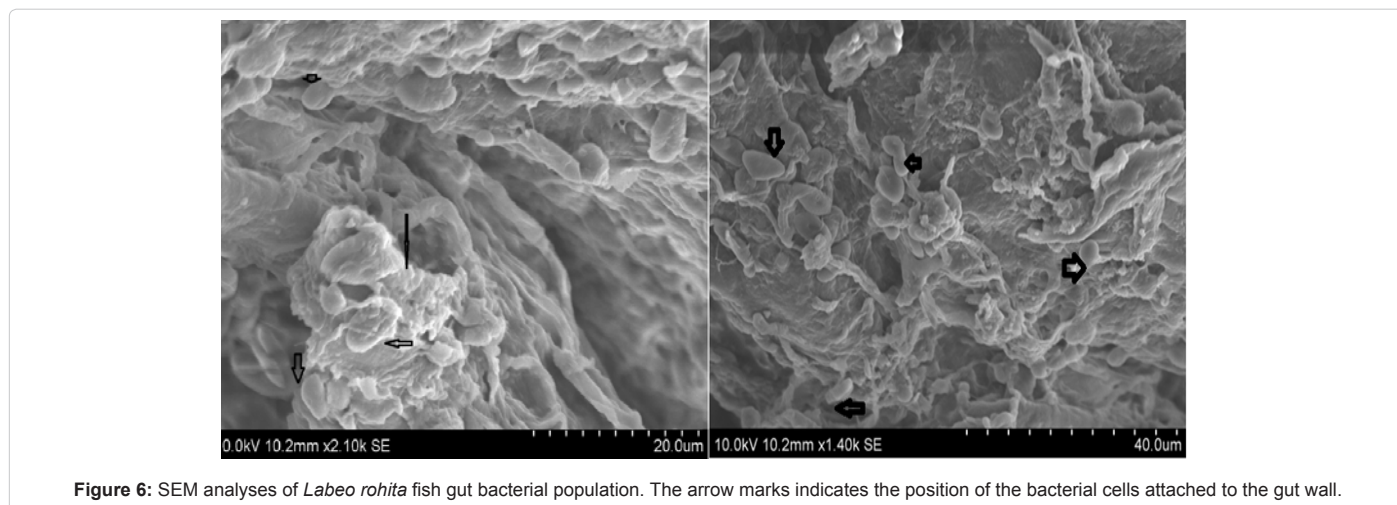
Figure 6: SEM analyses of *Labeo rohita* fish gut bacterial population. The arrow marks indicates the position of the bacterial cells attached to the gut wall.

diet for the fish. In diet 2 the microwave heat treatment process would have untangled the closely knit collagen structure present in ANFL thereby facilitating its easy uptake from the feed. In the diet 3 and 4, the native protein present in raw ANFL reduced assimilation; therefore, both the bacterial population and enzyme activity remained dormant (Table 2 and Figure 1). The gut extract of diet 5 fed fish showed maximum protease activity than control fish meal based feed. Elevated bacterial population with synchronized increase in protease activity of *Labeo* gut fed with fermented ANFL reveals the adaptability of rohu to new protein source. It is further noteworthy to state that diet 5 showed the maximum number of proteolytic bacterial count when compared to all other diet fed fish groups (Table 2). The probable reason could be the introduction of heavy bacterial population exogenously to the gut from the feed containing fermentation bacteria. The fermentation bacteria help the host in digestion of food and give less chance for the pathogens to colonize the gastrointestinal tract. Diet 6 fed fish groups also exhibits protease activity higher than diet1, 3 and 4 but lower than the diet 5. Cellulase, amylase and lipase activities of the gut extracts in all the experimental fish were found to be more or less stable due to the similar feed components in the formulated diet.

Since the experimental diets contain carbohydrate sources such as wheat flour and rice bran considerable population of amylolytic bacterial strains were detected. The highest amylase producing strain RF6 (186 U) was isolated from the experimental diet 1. Similar results have been reported by previous researchers with carbohydrate based feed [24,42,43].

Microbial intestinal cellulase activity has been detected earlier in carp [44]. grass carp [43]. and in [38]. fingerlings. Quantitative assay of the selected strains showed relative cellulase activity due to the presence of natural complex cellulose containing plant products like wheat flour and rice bran in the diet (Figure 2 and Table 3). Hence, the cellulase activity was low in the gut extract compared to that of the proteases due to the formulation of protein rich diets. However, the strains FF2 and FF4 were observed to have the highest activity among all other isolates for cellulase production. These bacterial strains present in the gut might indigenously posses the cellulolytic activity or may have derived due to ingestion of rice bran (plant source).

Although fish have an endogenous source of protease in their digestive tracts, not much attention has been paid to the microbial source of protease in fish. Ghosh et al.[15] suggested that *Bacillus*

circulans, *B. pumilus*, and *B. cereus*, isolated from the alimentary canal of *Labeo rohita* fingerlings were good producers of proteolytic enzymes, though they did not quantify the enzyme activity. Since the experimental diets were protein rich the bacterial strains as well as the gut extracts showed higher protease activity than other enzymes and this corroborates with the prior report wherein increased proteolytic enzyme activity in common carp were observed with bovine-trypsin based diet [45]. Kawai [86] reported adaptive changes in the proteolytic enzyme in common carp (*Cyprinus carpio*) in relation to the type of the diet. The occurrence of proteolytic bacteria in the gut of the isolated fish species seems to support the presence of a diet dependent microbial population [37]. Further these investigations have suggested that microorganisms have a beneficial effect in the digestive processes of fish. A number of studies on the use of proteases to improve the digestibility of feed ingredients in poultry, pigs and cattle have been published. In broiler chickens, the addition of protease to lupin-based [89] or soya bean-based [90, 91] diets resulted in improved digestibility of protein.

The micro flora of the fish gut is generally culturable and occurrence of protease, amylase and cellulase and lipase producing bacterial population is noteworthy in the digestive tract of rohu. Digestive enzyme activities has also been reported earlier using *Bacilus sp.* containing probiotics in common carp [46]. *B. subtilis* in four live-bearing ornamental fishes [47]. *Lactobacillus spp.* [48] in beluga (*Huso huso*) and Persian sturgeon (*Acipenser persicus*) [49] and live yeast in European sea bass larva [50]. Cellulolytic and Amylolytic gut microflora has been reported earlier [4,38,40,92]. Protease producing organisms and their activity was observed in the gut of the six ANFL formulated diets fed groups and is highest in the fermented ANFL fed *Labeo rohita* which may be due to the presence of fermentation facilitating bacterial strains. Furne et al. [87] observed that it is possible to predict the ability of a species to utilize different nutrients based on its digestive enzyme profile. The bacterial diversity in the intestines of abalone fed with artificial food was higher than in individuals fed with sea algae [87] and differed between puffer fish fed a natural diet and those fed with an artificial diet [71,87]. The information generated from the present study might contribute towards the research of diet dependent change of micro biota in the gastrointestinal tract of *Labeo rohita* as well as the low cost feed formulation process utilizing the tannery solid waste as protein source. The study also indicates that there is a distinct microbial source of the digestive enzymes – amylase, cellulase, lipase

and protease, apart from endogenous sources in fish gut through ANFL administered feed which helps the fish to digest the nutrients well and lead to increase in the total enzyme producing microbial population.

The information generated from the present investigation might contribute to the incorporation of these bacteria in commercial aquaculture as supplement in formulated fish feed or in form of bacteria biofilm to achieve colonization in the fish gut at a higher degree. However, further research involving potent bacterial strains should be conducted for evaluating their efficacy under actual farm conditions. The results are also in agreement with the previous research findings that type of food can influence the enzymatic activity [81,92]. and the bacteria, initially introduced with food, further become adapted to particular gastro-intestinal environmental conditions, and develop as a distinct or transient community of facultative aerobic which, being metabolically active, play an active role in digestive processes [51-55]. However, the influence of these bacterial populations on the fish productivity is not known and requires further investigation.

In fish, data pertaining to digestive enzyme activity and profiles have helped overcome nutritional problems associated with formulation of artificial diets that best meet an animals' nutritive capability [86]. The results also present a scope for fish nutritionists to utilize the enzyme-producing bacterial isolates as a probiotic in formulating cost-effective aquafeeds, especially for the larval stages when the enzyme system is not efficient. However, further investigations are required to know about the metabolic pathways used by these microorganisms in the alimentary tracts of fish, to explain the lifelong host–microbe homeostasis.

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